494 MONOLAYER EXPANSION MODALITIES INFLUENCES 3D CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS SEEDED IN COLLAGEN SPONGES

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Purpose: Cartilage engineering requires a large population of cells that are seeded to scaffold. To this end, monolayer expansion is often used to increase cell number. The objective of this study was to test the primary influence of culture medium during monolayer expansion on the secondary chondrogenic differentiation and ancillary extracellular matrix (ECM) production of mesenchymal stems cells (MSCs) seeded in collagen sponges.
Methods: After isolation from human bone marrow, MSCs were cultivated in monolayer with or without bFGF from passages 0 to 2. At passage 3, just before MSC seeding into sponges, cells were divided into 5 groups: (1) differentiation medium (DM), (2) DM + chondrogenic supplement, (3) DM + bFGF, (4) DM + bFGF + chondrogenic supplements, (5) DM + bFGF + TGFβ1. After passage 3, MSCs were then seeded in collagen I sponge and cultivated for 28 days in vitro. Each group was cultivated with 8 different mediums containing ITS (Insulin Transferin Selenium) or FBS (fetal bovine serum) supplemented or not with TGFβ1 or BMP-2. ECM production and chondrogenic differentiation of MSCs during 3D differentiation were evaluated at D28. Chondrogenic gene expression (COMP, Aggrecan, Versican, Coll 2, Coll 1, Coll 3, Sox9, Osteocalcin, Alkaline Phosphatase) was investigated by qPCR. Newly synthesized ECM was assessed histologically and immunohistochemically (Coll 1 and Coll 2).
Results: Medium composition during 2D expansion strongly influenced ECM production by MSCs during 3D culture in sponges: FGF supplementation alone or in combination (chondrogenic supplements or TGFβ1) increased ECM production inside the biomaterial. In 3D conditions, growth factor (TGFβ1 or TGFβ1+BMP) combined with ITS increased the quantity and quality of ECM. Conversely, BMP2 alone had no influence when added to ITS or FBS alone. In fact, TGFβ1 alone or the sequential exposure of TGFβ1 (D3-D14) followed by BMP2 (D15-D28) promoted both chondrogenic expression (COMP, Aggrecan, Coll 2++) and chondral phenotype as synthesized matrix contained proteoglycans as demonstrated histologically and by Coll1 + Coll2 immunostaining. In addition, polarized light microscopy depicted collagen network in the ECM. In contrast, when growth factors were associated to FBS during 3D cultures, there was a detrimental influence of this combination on chondrogenic differentiation of MSCs.
Conclusions: For cartilage engineering, chondrogenic differentiation of MSCs seeded in collagen sponges is promoted by a previous exposure to bFGF during expansion phase and by the contact to TGFβ1 alone (D3-D28) or switched by sequential BMP2 (D15-D28) during differentiation.

495 BONDING OF MENISCAL TISSUE WITH CELLULAR FIBRIN GLUE: A NUDE MOUSE STUDY

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Purpose: Meniscus repair is a current clinical challenge. Menisci play a fundamental role in knee biomechanics, but they lack intrinsic regenerative properties. Consequently, when a tear occurs and the meniscus is removed surgically, even partially, crucial changes in knee homeostasis take place, often leading to the development of early osteoarthritis. In the last decades tissue engineering approaches have been advocated to improve the reparative processes of joint tissues. The aim of this study is to assess the capacity of isolated chondrocytes embedded in fibrin glue to promote bonding of meniscal slices in a nude mice model.
Methods: A swine chondrocytes-fibrin glue suspension was utilized as a biologic glue to improve bonding between two meniscal slices obtained from swine menisci. Tissue engineered tri-phasic sandwiches were obtained combining two meniscal slices with cellular fibrin glue and then wrapped with acellular fibrin glue. Radial slices were harvested from pig menisci and regularized with a scalpel. Chondrocytes were isolated from articular cartilage of pigs by collagenase digestion and then resuspended in fibrinogen solution. Cell solution was placed atop of a meniscal slice. The thrombin was added to form a fibrin glue gel embedding chondrocytes. During polymerization of fibrin glue, a second meniscal slice was put over the fibrin glue in order to form the tri-phasic sandwich. We prepared also control samples with acellular fibrin glue between the meniscal slices in order to evaluate the role of cells in the bonding process. All samples were wrapped in an acellular fibrin gel and then implanted in the subcutaneous tissue of nude mice for four weeks.
Results: At the end of the fourth week from implantation, samples were retrieved, macroscopically analyzed, tested for gross bonding and processed for histological evaluation. The fibrin glue embedding the samples was almost absorbed and the remnants appeared reorganized into a neo-capsule wrapping each sample, rich of neovessels. Moreover, the meniscal slices did not show any shrinkage or signs of digestion demonstrating the effectiveness of the fibrin gel embedding as a “shield” against metalloproteinases digestion, while the fibrin gel itself appeared rich of cells likely deriving from the host animal. The gross bonding between the meniscal slices was tested with a pair of forceps and demonstrated a firm adhesion between the two slices in all the experimental samples. On the other hand, none of the control samples showed any sign of bonding. Histological evaluation (H&E) demonstrated the presence of an hypercellular fibrocartilaginous tissue at the interface between the slices. Interestingly, some penetration buds were present inside the meniscal slices coming from the cellular fibrin gel. No penetration buds and no cellular tissue was found in the control samples. SEM confirmed the presence of a continuous tissue in the interface between the meniscal slices in the experimental samples.
Conclusions: These results demonstrated the potential of this model for improving meniscal bonding and confirmed the importance of cells in the bonding process of tissues. However, further orthotopic studies in a large animal model are needed to evaluate its feasibility in clinical practice.

496 ENGINEERING AN OSTEOCHONDRAL PLUG: ANALYSIS OF CHANGES FROM THE IN VITRO TO THE NUDE MICE CULTURE

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Purpose: Articular cartilage has a poor intrinsic regenerative potential. As a matter of fact, when a lesion occurs, the repair tissue is often fibrous, having insufficient biomechanical properties, which could frequently lead to the development of early osteoarthritis when physiologic mechanical forces act on this repair tissue. In the last decade, tissue engineering approaches addressed this topic with many cell-seeded biomaterials and some of them are currently used in clinical practice. The aim of this study was to assess the optimal time of in vitro development of ostechondral plugs before implantation in subcutaneous pouches of nude mices.
Methods: Osteochondral cylinders were developed combining a fresh chondrocytes-fibrin glue gel composite with a calcium-phosphate scaffold. Chondrocytes were isolated from articular cartilage of pigs by collagenase digestion and resuspended in fibrinogen solution. The thrombin was added to form a fibrin glue gel composite with cells. During polymerization of fibrin glue, the scaffold was put over the fibrin glue. Composites were placed in standard culture conditions and then retrieved after one, three and five weeks. At the end of the in vitro culture, half of the samples were analyzed and the other half implanted in subcutaneous pouches of nude mice for four weeks. Gross evaluation, histology, immunohistochemistry, biochemical analyses for DNA (Picogreen) and GAGs (DMB), biomechanical evaluation (compression and shear properties).
Results: Samples cultured in vitro for 1 week only demonstrated a shrinkage at the end of the in vivo culture while samples cultured in vitro for 5 weeks maintained their original volume. Histological evaluation showed the presence of cartilage-like tissue maturing within the fibrin glue gel, and a macroscopic penetration of the cellular fibrin glue into the pores of the calcium phosphate scaffold. Moreover, GAGs seem to adhere to and imbue the hard scaffold, determining areas of integration between the calcium-phosphate and the cellular fibrin glue. Immunohistochemical assay demonstrated that collagen type 2 was present either at 1 week and 5 weeks with a more strong staining at 5 weeks and after in vivo culture. DNA quantisation demonstrated an increase in total DNA quantity

in the specimens after 5 weeks of in vitro culture compared to those cultured for 1 week only ($p < 0.05$). DMB assay demonstrated a more abundant synthesis of GAGs in the specimens cultured for 5 weeks ($p < 0.05$). Biomechanical compression test demonstrated that Young's modulus (E) of the specimens cultured for 5 weeks was equal to that of the unseeded fibrin glue. Shear properties analysis demonstrated higher elastic properties (G′) than those of the unseeded fibrin glue. Biochemical and biomechanical properties showed an increase after in vivo culture.
Conclusions: We noticed an increase in the collagen type 2 synthesis, in the biochemical, compression and shear properties over the experimental times, suggesting that culturing the samples before implantation can give the surgeon a more developed and resilient construct. Moreover, samples cultured for 1 week only in vitro showed an important decrease in volume after in vivo culture, confirming the importance of pre-culturing the engineered tissue before implantation. However, this model needs to be validated in an in vivo orthotopic environment in order to evaluate its behaviour under physiologic load bearing stimuli.

497 CHONDROGENESIS OF HUMAN MESENCHYMAL STEM CELLS IN A SELF-AGGREGATING CULTURE MODEL AND THE EFFECTS OF HYDROSTATIC LOAD

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Purpose: Cartilage damage is a clinical dilemma with much attention to cartilage tissue engineering and a focus on bone marrow-derived mesenchymal stem cells (MSCs) given their potential for clincial relavance. In this study, we attempt to produce a cartilage-like biomass using a self-aggregating suspension culture model whereby with chondrocytes the chondrogenic phenotype is sustained without the use of a scaffold. Our study's purpose was to question if a self-aggregating scaffold-less culture model could serve as a method to promote chondrogenesis and could it be affected by a mechanical load.
Methods: MSC were derived from human bone marrow with IRB approved and cultures of adherent cells were expanded for no more than 3 passages. Cultures were established with 1×10^6 cells/ml, maintained in 24 well dishes coated with poly-HEMA in either control or chondrogenic medium (CM). Cultures were maintained for up to 6 weeks (preconditioning) in DMEM with 10% FBS, with antibiotics and 50 ug/mL ascorbate (Control) or DMEM with 10 uL/mL ITS+, 50 ug/mL ascorbate, 40 ug/mL proline, 10 ng/mL TGF-β, and 0.1 μM dexamethasone (CM). For each week of culture 3 samples were hydrostatically loaded and 3 were controls; loaded cultures were placed in vials with a flexible membrane inset and held in a customized bioreactor attached to a hydraulic piston connected to an Instron. Cyclical loading ranging from 0.5−5 MPa was applied for 3 hrs at 0.1 Hz. This process was repeated 3 times on successive days followed by a two day rest before the RNA isolated and histology. Levels of mRNA were determined of collagen type II, aggrecan, COMP, iNOS, MMPs, and GAPDH.
Results: MSC grown in chondrogenic medium using our self aggregating culture model, form a mass and even after only 2 wks in culture greatly increased the expression of cartilage-specific markers CII, aggrecan, and COMP. Expression increased exponentially for the first 4 wks. The idea of pre-load conditioning followed the logic that MSCs must develop like neonatal cartilage prior to addition of physiological load forces. The histochemistry revealed marked ECM accumulation and a linear time-dependent decrease in cell-to-matrix ratio similar to developing cartilage. CM significantly ($p < 0.05$) increased the wet weight of the biomasses 8-fold over control while proliferation was only 2-fold control. Hydrostatic loading did not consistently increase cartilage gene expression as we have shown for chondrocytes, but rather decreased ECM gene expression particularly with shorter preconditioning time points although some trends was observed of up-regulation after 4 wks of preconditioning. Telling of cell stress iNOS was significantly ($p < 0.05$) upregulated in loaded samples. No increased of iNOS expression was seen in loaded chondrocytes (which responded well to loading) suggesting the correlation of the negative response to load is related to a non-optimal cell-matrix ratio.
Conclusions: We demonstrate that using a high-density self-aggregating suspension culture model MSC can be differentiated, and will quickly produce a biomaterial with features consistent with a hyaline cartilage phenotype. Our results show that MSCs may need to establish an appropriate cell-matrix ratio before withstanding even a physiological load. Our results suggest that the benefit of loading may be dependent on the accumulation of ECM and longer preconditioning times are being examined. This study describes a bioreactor model without foreign material or scaffold in which BMSC can form a cartilage-like biomass and represents a promising and simple methodological approach to cartilage tissue engineering.

498 THE OXIDATIVE METABOLISM INDUCED DURING MONOLAYER EXPANSION OF CHONDROCYTES IS PARTIALLY MODULATED BY OXYGEN SUPPLY

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Purpose: Autologous Chondrocyte Implantation for cartilage repair requires that isolated cells are expanded in monolayer before re-implantation to the defect. This strategy is limited by the loss of native cell phenotype *in vitro*. Here we test the hypothesis that ROS generation by chondrocytes cultured under atmospheric O_2 induce the switch from the phenotypic glycolytic metabolism to an oxidative one.
Methods: Bovine chondrocytes were seeded into flasks at 2.3×10^4 cells$\times$cm^{-2}. These were distributed between standard incubator conditions (20% v/v O_2) and two reduced O_2 conditions, 5% and 2% O_2. A glove-box integrated with the incubators (BioSperix, USA) provided uninterrupted control of the oxygen atmosphere. All media (DME+10% FBS) and reagents were pre-equilibrated with the specified atmosphere. Additional flasks were transferred from 20% O_2 to 2% O_2 after 7 days and assessed at day 14. To assess role of ROS, a non-lethal dose of H_2O_2 (10 μM) was added to flasks under 5% O_2.
At days 0, 3, 7 and 14 the O_2 consumption rate (OCR), glycolytic rate, and oxidative stress of all chondrocytes were assessed as a suspension in 200 μM O_2. 320 μl aliquots were transferred to the wells of an O_2 biosensor plate (BD Biosciences, Oxford, UK) in triplicate with 200 μM and 0 μM O_2 reference conditions and [O_2] recorded from the sealed plate at 10 minute intervals. 3 μM of the uncoupler CCCP or 2 μg.mL^{-1} oligomycin indicated maximal oxidative capacity and non-F_1F_0 mediated OCR respectively. Glycolytic rate and oxidative stress were determined from lactate and 8-OHdG in the media over 30 min. Total protein per cell was measured using BCA assay. Data were collected from 3 independent experiments. Error bars represent s.d.
Results: Proliferation was highly dependent on O_2 with 10.2, 5.9 and 4.6-fold increased cell yield under 20%, 5% and 2% O_2 respectively. Glycolytic rate and total protein$\times$cell^{-1} rose from initial values of 161±51 fmol$\times$cell$^{-1}\times$h^{-1} and 61±4 pg$\times$cell^{-1} at day 0 to 1063±141 f mol$\times$cell$^{-1}\times$h^{-1} and 204±7 pg$\times$cell^{-1} after 14 days under 20% O_2. However, no significant difference could be attributed to incubator O_2.
OCR values, all tested in 200 μM O_2, were dependent on the incubator O_2 atmosphere during expansion (fig 1). The OCR of cells expanded under 2% O_2 was 0.56 that of cells cultured in 20% O_2 at day 14. At 39±5 f mol$\times$cell$^{-1}\times$h^{-1} this was still 14X the values observed at day 0. Maximal oxidative capacity was enhanced proportionately, representing biosynthesis of mitochondria.

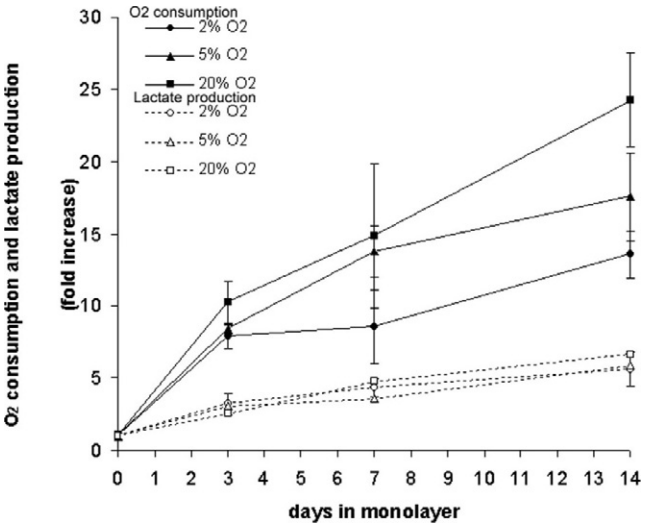

Figure 1.

Expanded cells exhibited increased 8-OHdG compared to day 0. This marker of oxidative stress was also significantly increased in cells expanded under 20% O_2 compared to physiological O_2 (fig 2).